

*Running title:*  
Electro-olfactogram

ORIGINAL CONTRIBUTION

## Olfactory training changes electrophysiological responses at the level of the olfactory epithelium

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## SUMMARY

**Background:** “Olfactory training” (OT) is known to increase olfactory performance in healthy subjects and patients with post traumatic or post viral olfactory loss. Morphological correlates such as olfactory bulb volume increase and gray matter changes suggest central changes in olfactory brain areas following olfactory exposure. Some evidence from animal studies indicates peripheral changes upon OT whereas no such data exists in humans. This study explores the question whether changes in olfaction following OT are associated with alterations of the electro-olfactogram (EOG) derived from the olfactory epithelium.

**Methodology/Principal:** We compared electrophysiological EOG responses to a rose-like odor (phenylethyl alcohol, PEA) and to an unpleasant odor (H<sub>2</sub>S) in patients and controls. EOGs were recorded in smell impaired patients before and after OT for a period of 4- 6 months.

**Results:** EOG recordings following PEA and H<sub>2</sub>S stimulation were significantly more often obtained in controls than in patients. OT was associated with a significantly higher number of EOG recordings.

**Conclusions:** OT is associated with an increase in EOG responses implicating stimulus- induced plasticity to start at the level of the olfactory epithelium.

Key words: olfaction, olfactory training, olfactory receptors, nose, electro-olfactogram

## INTRODUCTION

Exposure to odors may improve olfactory function. Repeated exposure to odors in healthy subjects has been shown to increase olfactory sensitivity<sup>(1-3)</sup>, or to improve the recovery of patients with post viral olfactory loss<sup>(4,5)</sup> and prevent olfactory deterioration in older people<sup>(6)</sup>. Further on, patients with Parkinson's disease performing "olfactory training" (OT) were shown to improve in their olfactory ability<sup>(7)</sup>.

Few morphological correlates for OT effects have been described to date. At the level of the olfactory bulb OT has been shown to increase its volume in healthy volunteers<sup>(8)</sup>. In addition, changes in brain connectivity have been shown to be associated with OT<sup>(9)</sup>. And at the level of the olfactory epithelium (OE) there is limited evidence that OT can induce changes; in rats electrophysiological responses from the OE were found to increase in response to odor exposure<sup>(10)</sup>.

Thus, assuming that olfactory input manipulation induces changes at the OE in humans, we set out to explore the influence of repeated exposure to odors on electro-olfactogram (EOG) recordings in patients with olfactory loss. EOG are generator potentials of olfactory receptor neurons in response to chemical stimuli; they pose a comprehensive way of providing neural information from the peripheral olfactory level<sup>(11-16)</sup>.

## MATERIALS AND METHODS

The study was performed in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of the TU Dresden (application number EK12012006).

### *Participants*

Patients were randomly recruited from the Smell and Taste Clinic at the Department of Otolaryngology after applying below mentioned criteria. Informed consent was provided by each participant. Following inclusion and exclusion criteria were applied:

The study included only participants above the age of 18 years. The patient group contained patients with post traumatic, post infectious and idiopathic smell loss whereas participants from the control group had no impaired sense of smell reflected in normosmic test results in the Sniffin' Sticks test. Excluded from the study were those patients or participants with acute or chronic sinusal disease, chronic disease like Parkinson's disease or chronic renal failure associated with smell disorders, pregnant and lactating women.

With regard to those criteria a total number of 65 participants could be included in the study out of which 15 were anosmic ( $\bar{X}$  age: 49, SD: 11.6), 23 hyposmic ( $\bar{X}$  age: 55, SD: 11.2) and 27 normosmic ( $\bar{X}$  age: 49, SD: 11.5). OT was performed only in smell impaired patients and included were only those with a complete pre and post training status (23 out of 38).

### *Olfactory testing*

Olfactory testing was performed in 27 healthy participants and 38 patients (15 anosmic, 23 hyposmics) at baseline using the Sniffin' Sticks test. Out of 38 patients only 23 completed OT and underwent additional olfactory testing 4-6 months after OT.

The Sniffin' Sticks test consisted of three subtests<sup>(17)</sup>. In the odor threshold task subjects had to detect the odorized (phenylethylalcohol, PEA) pen among three samples with the other two pens containing odorless propylene glycol. PEA concentration was increased if the subjects' answer was wrong and decreased if the given answer was correct. In the odor discrimination task again a triplet of pens was presented to the subject who had to discriminate one different odor from two identical odors. Regarding the odor identification task, subjects had to smell a single odorous pen and choose the correct answer from a list of four descriptors. The sum of the three tests accounted for the TDI- score with a maximum of 48 points (each subtest 16 points). Normosmia was set at 30.5 points or more in the composite TDI score, whereas hyposmia was diagnosed between 16.5 and 30.5 points and anosmia below 16.5 points<sup>(18)</sup>.

### *OT*

Twenty-three patients completed OT for 4-6 months using four different odors (eucalyptol, citronella, eugenol, phenyl ethyl alcohol). Odors were presented in brown glass jars containing a cotton pad soaked with 4 ml of odor. Patients had to smell twice in the morning and twice in the evening each odor for 10 seconds.

### *EOG recordings*

All subjects were exposed to a specific breathing technique (velopharyngeal closure<sup>(19)</sup>) prior to the recordings. This technique avoids respiratory flow inside the nasal cavity during mouth breathing through lifting the soft palate and closing the nasopharynx. For chemical stimulation, an olfactometer was employed delivering the odorants to the left or right nostril without altering mechanical or thermal conditions at the stimulated mucosa<sup>(19,20)</sup>. Therefore, pulses of the stimulants were embedded in a constantly flowing air stream with controlled temperature (36.5° C) and humidity (80% RH). The air stream was led into the nasal cavity with a flow rate of 6L/min by way of a teflon tubing (8 cm length, 3 mm inner diameter). Two thirds of the maximum stimulus concentration were reached at the olfactometer's outlet within 20 ms<sup>(19)</sup>. For stimulation phenyl ethyl alcohol (40% v/v PEA) and hydrogen sulphide (8 ppm H<sub>2</sub>S) were chosen which are regarded to specifically activate the olfactory system<sup>(21-23)</sup>. In addition, CO<sub>2</sub> (60% v/v; air liquide, Düsseldorf, Germany) was used at concentrations which did produce clear trigeminally mediated sensations (e.g., burning or tickling).

White noise of approximately 50 dB SPL was used to mask switching clicks of the stimulator.

EOG was recorded from both nostrils by means of a tubular electrode filled with Ringer-agar (1%) which contained a silver chlorided silver wire (impedance  $\leq 10 \text{ k}\Omega$  at 1 kHz<sup>(11)</sup>, outer diameter 0.8 mm). The electrode was placed under endoscopic control (Richard Wolf, Knittlingen, Germany; outer diameter 1.9 mm) approximately 7 cm deep into the nasal cavity until the contact between electrode and mucosa had been established within the olfactory cleft. The recording site was marked in a sketch of the human nasal cavity<sup>(24)</sup>.

The electrode was then stabilized by means of adjustable clips on a frame similar to lensless glasses<sup>(12)</sup>. After a response had been established in return to stimulation with either PEA, H<sub>2</sub>S or CO<sub>2</sub> the three stimuli were randomly applied 2-16 times at an interval of approximately 60 s. Mucosal potentials were amplified, filtered (band pass 0.01-15 Hz; SIR, Röttenbach, Germany), and digitized (sampling frequency 125 Hz, segments of 8192 ms). After averaging, maximum EOG amplitudes (P1, N1, P2, p1n1, n1p2) and latencies (lp1, ln1, lp2, p2p1) were analysed for each individual. The clear delineation of a large negative response (N1 amplitude or N1 proof) was interpreted as a peripheral odor evoked potential or the existence of an EOG potential in response PEA or H<sub>2</sub>S.

### *Statistical Analysis*

Using SPSS 23.0 (SPSS Inc. Chicago, Ill., USA) data were statistically analysed by means of t-test for independent samples (because EOGs were obtained in inhomogeneous groups of people) to investigate differences between patients and controls for the different stimulus qualities. Using chi<sup>2</sup>-tests we also looked at differences between groups in the number of responses.

## RESULTS

### *Olfactory performance*

There was no significant difference in age distribution between healthy, hyposmic and anosmic participants ( $p = 0.10$ ) using analysis of variance. Comparing the three groups regarding their olfactory performance, a highly significant difference in TDI score and all subtests appeared ( $p_s < 0.001$ ) at baseline. While healthy participants showed an average TDI score of 34.7 points (SD  $\pm 2.8$ ), hyposmic patients had a score of 23.6 (SD  $\pm 4.0$ ) and anosmic patients of 11.5 points (SD  $\pm 3.3$ ).

Patients ( $n = 23$ ) performing OT showed an average TDI score of 19 points (SD  $\pm 7.9$ ) at baseline. A significant improvement in olfactory performance could be shown in the identification task ( $t [22] = -3.59$ ,  $p = 0.002$ ,  $M = 1.7$ ). But no such difference could be demonstrated for the composite TDI, discrimination or threshold score ( $p > 0.05$ ). A clinically relevant improvement of olfaction, which was defined as an improvement of 6 points within the TDI score<sup>(25)</sup>, was achieved in 8 (35%) patients.

### *EOG recordings*

Comparing healthy subjects' and patients' recordings at baseline

EOG recordings were attempted in 27 healthy subjects (20 women, 7 men, mean age 49 years; SD: 11.5) and 38 patients with olfactory loss (15 anosmic [6 women, 9 men  $\emptyset$  age: 49, SD: 11.6], 23 hyposmic [17 women, 6 men,  $\emptyset$  age: 55, SD: 11.2]). EOGs could be recorded in all subjects, however, not all subjects exhibited responses to all stimulus qualities (table 1).

No significant differences in EOG amplitudes (P1, N1, P2, p1n1, n1p2) or latencies (lp1, ln1, lp2, p2p1) emerged between the two groups ( $p_s > 0.05$ ). However, using the Chi-Square test, we could demonstrate that the N1 amplitude and therefore the stimulus response to PEA and H<sub>2</sub>S in the left nostril was significantly more often obtained in healthy participants than in patients (PEA:  $\text{Chi}^2 = 29.13$ ,  $p < 0.001$ , H<sub>2</sub>S:  $\text{Chi}^2 = 13.63$ ,  $p = 0.001$ ). No such differences emerged in response to CO<sub>2</sub> stimulation ( $\text{Chi}^2 = 1.73$ ,  $p = 0.42$ ). Similarly, statistically significant differences could be detected for the right nostril in response to PEA and H<sub>2</sub>S but not for CO<sub>2</sub> stimulation (PEA:  $\text{Chi}^2 = 11.10$ ,  $p = 0.004$ , H<sub>2</sub>S:  $\text{Chi}^2 = 11.74$ ,  $p = 0.003$ , CO<sub>2</sub>:  $\text{Chi}^2 = 2.22$ ,  $p = 0.33$ ). The number of patients showing EOG recordings to different stimuli divided by each nostril is shown in table 1.

Comparing patients' recordings before and after OT

Because 15 smell impaired patients were excluded from the study due to mal compliance or no-show, EOG recording was compared in 23 (12 women, 11 men;  $\emptyset$  age: 54 years, SD: 12.6) out of 38 patients before and after OT. No significant differences in EOG amplitudes (P1, N1, P2, p1n1, n1p2) or latencies (lp1, ln1, lp2, p2p1) emerged between the groups ( $p_s > 0.05$ ). However, in terms of the number of responses to PEA and H<sub>2</sub>S stimulation in the form of N1 proofs, following differences could be shown: in response to PEA and H<sub>2</sub>S, both specific activator of the olfactory system, a significantly higher number of EOGs could be recorded after OT (no. of EOGs before: 34, after: 62,  $\text{Chi}^2 = 26.1$ ,  $p < 0.001$ ). Considering specifically the sight of recording and the responses to each odor, a significantly higher number of responses to H<sub>2</sub>S could be obtained within the right nostril after OT as compared to baseline (H<sub>2</sub>S:  $p = 0.001$ ). But no such difference could be demonstrated on the left side (H<sub>2</sub>S:  $p =$

0.39). Regarding the responses to PEA stimulation, a higher number of EOG responses with a clear statistical tendency could be demonstrated in the left nostril after OT (PEA: l:  $p = 0.07$ , re:  $p = 0.15$ ) (Fig1). Upon CO<sub>2</sub> stimulation, no such differences emerged on both sides after OT (CO<sub>2</sub>: l: n.s., re:  $p = 0.22$ ) (table 2).

As mentioned above, clinically relevant improvement with a TDI score increase of 6 points was achieved in 8 out of 23 patients (35%). Comparing improved patients (n = 8) with those showing no relevant improvement in TDI score (n = 15) no significant differences appeared in terms of the number of EOG recordings following PEA, H<sub>2</sub>S and CO<sub>2</sub> stimulation ( $\chi^2 < 2.81$ ,  $p > 0.09$ , table 3).

## DISCUSSION

Major results found in our study include (1) that EOG recordings following PEA and H<sub>2</sub>S stimulation were significantly more often obtained in healthy participants than in anosmic and hyposmic patients. (2) This finding could consistently be demonstrated in both nostrils of healthy subjects and patients. Further, (3) OT was associated with a significantly higher number of EOG recordings in responses to H<sub>2</sub>S and PEA stimulation obtained from the olfactory mucosa of both nostrils but also in response to H<sub>2</sub>S in the right nostril. (4) A statistical tendency could be obtained in response to PEA stimulation showing a higher number of responses in the left nostril after OT. (5) No differences in electrical activation appeared in response to CO<sub>2</sub> between healthy subjects and patients as well as (6) no differences emerged after OT as compared to baseline. Finally, (7) no significant differences in the number of EOG responses emerged between those patients experiencing a clinically relevant improvement after OT and those who did not.

At first, the current results suggest that olfactory impairment of various entities seems to start as early as at the level of the olfactory receptor neuron (ORN) which is reflected in a lower number of EOG recordings obtained from the olfactory mucosa of smell impaired patients. Both in post traumatic<sup>(26)</sup> and post viral olfactory<sup>(27)</sup> dysfunction the OE is known to be markedly disorganized and the olfactory receptors diminished in number. Therefore, with the electrophysiological changes demonstrated in our study, those histopathological alterations seem not only to present morphological but also functional changes at the level of the ORN. A significantly lower olfactory performance in our patients as compared to healthy participants demonstrates the functional alteration on a psychophysical level which is further in line with the diminished electrophysiological response activity shown in our study. Hence, for the first time, a decreased peripheral electrical activity could be demonstrated in humans with acquired olfactory loss.

Second, our present data shows that OT is associated, at least to some extent, with an improvement of electrical activity at the OE. This finding is in line with data existing from rats, where enhancement of the spatial activity pattern at the level of the olfactory mucosa was found following repeated exposure to odorants<sup>(10)</sup>. As suggested from previous work<sup>(28,29)</sup> odor stimulation leads to cell depolarization of the ORNs resulting in negative voltage transient which can be recorded from the mucosal surface. Hence, an improvement in EOG recording could indicate an increase in olfactory receptor expression or a higher affinity of those existing receptors caused by repeated short term odor exposure. While the turnover of ORNs in rodents is known to take 30-90 days<sup>(30,31)</sup> no data exists on the life span of human neurons. However, considering the OT duration of 4 -6 months, a quantitative increase in ORNs as a response to OT should additionally be taken into account. Because there was no control group to compare the results, we are finally not able to prove that the effects shown are caused by OT rather than spontaneous recovery. However, there is evidence in literature for the superiority of OT over spontaneous recovery in improving olfactory performance<sup>(5,32)</sup>. Therefore, we assume that the increase in obtained EOG potentials after training was related to repeated short term odor exposure.

Since the damage to the OE following trauma or viral infections does not follow the exact same pattern in both nostrils differences in regeneration between the two nostrils could appear, explaining the disproportionate improvement in electrical activity between the nostrils. Additionally, a significant

improvement in olfaction after OT could only be demonstrated for the identification task but not for the threshold or discrimination test which could also explain the overall limited improvement in electrical activity. But since we were able to record EOGs not only from hyposmic but also anosmic patients we could clearly demonstrate the existence of remaining functional ORNs in anosmics which is in line with findings of Rawson et al. demonstrating functionally mature ORNs in Kallmann syndrome patients with bilateral anosmia<sup>(33)</sup>.

Compared to PEA and H2S stimulation, no difference in EOG recording between healthy and dysosmic patients or between pre and post training status emerged upon CO<sub>2</sub> stimulation. Since CO<sub>2</sub> presents a purely trigeminal stimulus, the obtained recordings present trigeminally mediated potentials rather than olfactory potentials. Hence, OT did not show any effects on the trigeminal system as expected. There is evidence in literature showing a reduced trigeminal sensitivity in patients with acquired olfactory loss<sup>(34)</sup> which we could not find at least in the periphery. This reduced trigeminal sensitivity seems due to a lack of central nervous- interaction between the olfactory and trigeminal system whereas adaptive mechanisms seem to increase peripheral responsiveness<sup>(35)</sup>. Hence, in line with our data, a reduced trigeminal sensitivity in acquired olfactory loss seems not to emanate from the periphery.

In those patients showing a clinically relevant improvement of 6 points in the TDI score<sup>(25)</sup> after OT we could not obtain a higher number of EOG recordings as compared to those who have not improved. Probably a low number of subjects in both groups (8 vs. 15) could not expose the differences between the groups as seen in type two errors. But on the other hand, we could again clearly show the existence of electrical potentials and therefore functioning ORNs in anosmic and non-improved patients as demonstrated in Kallmann syndrome patients by Rawson et al.<sup>(33)</sup>.

Although the current study involved patients with various causes of olfactory loss the aim of the study was not to study these different groups. As a consequence, the number of patients in the 3 groups of patients with idiopathic, postinfectious and posttraumatic olfactory loss was too small (in total n=23) to allow for meaningful statistical comparisons between these groups in relation to the effects of OT. Future studies, however, will focus on the possible differences in EOG responses in patients with various causes of olfactory loss in relation to changes in olfactory function.

In conclusion, we could show for the first time, that olfactory disorders were associated with a decreased peripheral electrical response. OT on the other hand was, at least to some extent, associated with a stimulus- induced plasticity at the level of the ORNs. Therefore, OT in humans seems not only to affect olfactory processing at the central level as described so far but also at the level of the OE.

#### ACKNOWLEDGEMENTS

This work was supported by a grant from the ‘Deutsche Forschungsgemeinschaft’ to TH (DFG HU441/18-1). SCP was granted by the ‘Dresden Fellowship Program’ of the “Technische Universität” (TU) Dresden.

#### AUTHORSHIP CONTRIBUTION

TH: Contributed to study conception and design, analysis and interpretation of the data, drafting and critical revision of the manuscript

GS: Contributed to study conception, data acquisition and critical revision of the manuscript

AH: Data acquisition and critical revision of the manuscript

SCP: Contributed to analysis and interpretation of the data, drafting and revising of the manuscript

#### CONFLICT OF INTEREST

TH, GS, AH, SCP declare that they have no conflict of interest.

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TABLES

Table 1

EOG recordings in normosmic, hyposmic and anosmic participants

No. (%) of responses to left (l) /right (r) nostril	Normosmia n = 27	Hyposmia n = 23	Anosmia n = 15	<i>p</i> value
CO <sub>2</sub> (l)	25 (93%)	23 (100%)	14 (93%)	p = 0.42
CO <sub>2</sub> (r)	23 (85%)	19 (83%)	10 (67%)	p = 0.33
PEA (l)	26 (96%)	5 (22%)	8 (53%)	p < 0.001
PEA (r)	18 (67%)	12 (52%)	2 (13%)	p = 0.004
H <sub>2</sub> S (l)	24 (89%)	9 (39%)	9 (60%)	p = 0.001
H <sub>2</sub> S (r)	22 (81%)	10 (43%)	5 (33%)	p = 0.003

Table 2

## EOG recordings in patients performing olfactory training

No. (%) of responses to left (l) /right (r) nostril	Before n = 23	After n = 23	<i>p</i> value
CO <sub>2</sub> (l)	22 (96%)	23 (100%)	n.s.
CO <sub>2</sub> (r)	16 (70%)	20 (87%)	0.22
PEA (l)	7 (30%)	14 (61%)	0.07
PEA (r)	8 (35%)	14 (61%)	0.15
H <sub>2</sub> S (l)	11 (48%)	15 (65%)	0.39
H <sub>2</sub> S (r)	8 (35%)	19 (83%)	0.001
Total responses to PEA and H <sub>2</sub> S	34	62	< 0.001

Table 3

EOG recordings in patients showing/ not showing significant clinical improvement after olfactory training

No. (%) of responses to left (l) / right (r) nostril	Olfactory improvement < 6 points n = 15	Olfactory improvement ≥ 6 points n = 8	<i>p</i> value
CO <sub>2</sub> (l)	15 (100%)	8 (100%)	n.s.
CO <sub>2</sub> (r)	13 (87%)	7 (88%)	0.96
PEA (l)	11 (73%)	3 (38%)	0.094
PEA (r)	9 (60%)	5 (63%)	0.91
H <sub>2</sub> S (l)	11 (73%)	4 (50%)	0.26
H <sub>2</sub> S (r)	12 (80%)	7 (88%)	0.65

## TABLE LEGENDS

Table 1

Number (percentage, %) of obtained EOG recordings to different stimulus qualities in normosmic, hyposmic and anosmic participants.

Stimulus qualities: Carbon dioxide (CO<sub>2</sub>), Phenylethyl alcohol (PEA), Hydrogen sulphide (H<sub>2</sub>S)

EOG recording obtained nostril: left (l), right (r)

Table 2

Number (percentage, %) of obtained EOG recordings to different stimulus qualities in patients before and after olfactory training

Stimulus qualities: Carbon dioxide (CO<sub>2</sub>), Phenylethyl alcohol (PEA), Hydrogen sulphide (H<sub>2</sub>S)

EOG recording obtained nostril: left (l), right (r)

Table 3

Number (percentage, %) of obtained EOG recordings to different stimulus qualities in patients showing an olfactory improvement of 6 and more points in TDI score and patients showing less than 6 points of improvement after olfactory training

Stimulus qualities: Carbon dioxide (CO<sub>2</sub>), Phenylethyl alcohol (PEA), Hydrogen sulphide (H<sub>2</sub>S)

EOG recording obtained nostril: left (l), right (r)

## FIGURE LEGEND

## Figure 1

Top left: Participant with endonasal EOG electrode (thick black arrow) fixed to an adjustable clip on a lensless frame; stimuli are presented through thin cannula (thin black arrow). Bottom left: EOG recording from the olfactory epithelium; top right: EOG responses to olfactory stimuli before training separately for left- and right sided stimulation with phenyl ethyl alcohol or hydrogen sulphide (total: n=92 recordings); bottom right: EOG responses after training (total: n=92 recordings)